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Azotobacter chroococcum 7Fe ferredoxin

Two pH-dependent forms of the reduced 3Fe clusters and its conversion to a 4Fe cluster

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Ferredoxin from *Azotobacter chroococcum* has been studied by low-temperature magnetic-circular-dichroism and electron-paramagnetic-resonance spectroscopy. When aerobically isolated ferredoxin contains a [3Fe-4S] and [4Fe-4S] cluster. Anaerobic treatment with dithionite in the presence of ethanediol reduces the [3Fe-4S] cluster to give two spectroscopically distinct forms RI and RII which are reversibly interconvertible with a $pK_a \sim 7.5$. The higher-pH form, RII, has a high affinity for ferrous ion and converts readily to a $[4Fe\text{-}4S]^{1+}$ cluster, scavenging iron from the medium. The presence of the iron chelator EDTA inhibits this conversion.

The aerobic organisms *Azotobacter vinelandii* and *A. chroococcum* contain a ferredoxin (Yoch & Arnon, 1972; Shethna, 1970; Yates, 1970) which mediates electron transport to nitrogenase (Yates, 1977). The protein from *A. vinelandii* is air-stable when isolated aerobically and in this form has been characterized by X-ray crystallography (Ghosh *et al.*, 1981, 1982), by Mössbauer (Emptage *et al.*, 1980), e.p.r. (Sweeney *et al.*, 1975; Morgan *et al.*, 1984*b*), resonance Raman (Johnson *et al.*, 1983) and, in part, by m.c.d. spectroscopy (Morgan *et al.*, 1984*b*). These results show that, as aerobically isolated, the protein contains two different Fe-S clusters, one a [4Fe-4S] centre and the other a cluster of uncertain core stoichiometry [3Fe-*x*S] (Beinert & Thomson, 1983). The latter cluster gives a nearly isotropic $g = 2.01$ e.p.r. signal at liquid-He temperatures and is reduced by $Na_2S_2O_4$ at pH 7.5 to an even-electron, e.p.r.-silent paramagnetic state (Emptage *et al.*, 1980). Reduction of the protein with a 10:1 molar excess of $Na_2S_2O_4$ for 1h at pH 8.8 in 100mM-Tris buffer leads to the generation of an axial e.p.r. signal with *g*-values of 2.04 and 1.93, integrating to about 0.5 spins/mol of protein (Morgan *et al.*, 1984*b*). This has been assigned to a $[4Fe\text{-}4S]^{1+}$ cluster which, it is postulated, may arise either from the reduction of the $[4Fe\text{-}4S]^{2+}$ cluster present in the protein as extracted or, alternatively, by the conversion of the [3Fe-*x*S] cluster to a $[4Fe\text{-}4S]^{1+}$ cluster to generate a semi-reduced 8Fe ferredoxin ($[4Fe\text{-}4S]^{1+}[4Fe\text{-}4S]^{2+}$) (Morgan *et al.*, 1984*b*). The precedent for such a cluster conversion is provided by the activation of ox heart aconitase, which involves the conversion of a [3Fe-4S] to a [4Fe-4S] cluster (Kent *et al.*, 1982). This process is inhibited in aconitase by the presence of iron chelators such as EDTA (Kennedy *et al.*, 1983).

The structure of the [3Fe-*x*S] cluster in *A. vinelandii* Fd I is the subject of debate (Beinert & Thomson, 1983; Johnson *et al.*, 1983). The X-ray analysis points to a composition $[Fe_3S_3(S\text{-}Cys)_5X]$, where X is probably water (Ghosh *et al.*, 1982). The Fe_3S_3 core is nearly planar with an Fe–Fe distance of 0.41nm (4.1Å). By contrast, the structure of the oxidized state of the 3Fe cluster in *Desulfovibrio gigas* FdII and in ox heart aconitase has an Fe_3S_4 core stoichiometry with a Fe–Fe distance of 0.27nm (2.7Å) determined by X-ray absorption fine structure (e.x.a.f.s.) measurements (Antonio *et al.*, 1982; Beinert *et al.*, 1983). The Raman, Mössbauer, e.p.r. and low-temperature m.c.d. spectra of the oxidized 3Fe clusters in *D. gigas* FdII, aconitase and *A. vinelandii* Fd I are very similar in all proteins, suggesting that the core structures of the clusters are the same and unlike that deduced by X-ray crystallography. The only spectroscopic studies reported on the reduced state of the 3Fe clusters are the Mössbauer and low-temperature m.c.d. spectra of *D. gigas* Fd II (Beinert & Thomson, 1983), aconitase (Johnson *et*

Abbreviations used: Fd, ferredoxin, Pipes, 1,4-piperazinediethanesulphonic acid; Taps, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulphonic acid; m.c.d., magnetic circular dichroism.

al., 1984) and the Mossbauer spectra of the semireduced *A. vinelandii* Fd I (Emptage *et al.*, 1980).

We now report a study using low-temperature m.c.d. spectroscopy of the pH-dependence of the $Na_2S_2O_4$-reduced state of Fd from *A. chroococcum*. In the aerobically isolated state this Fd appears by c.d., m.c.d. and e.p.r. spectroscopy to be indistinguishable from *A. vinelandii* Fd I. The 3Fe cluster possesses, in the $Na_2S_2O_4$-reduced state, two forms distinguishable by m.c.d. spectroscopy, which we term RI and RII. The forms are pH-dependent, interconverting reversibly with a $pK_a \sim 7.5$. RII, formed above pH7.5, is spectroscopically identical with the $Na_2S_2O_4$-reduced 3Fe cluster in *D. gigas* FdII and in ox heart aconitase, pH8.5. Finally it is shown that state RII has a high affinity for ferrous ion, so that, with or without the addition of ferrous ion, the [3Fe-4S] reduced cluster is readily converted to a $[4Fe\text{-}4S]^{1+}$ cluster, resulting in a 8Fe ferredoxin in the semi-reduced state, ($[4Fe\text{-}4S]^{1+}[4Fe\text{-}4S]^{2+}$). RI does not so interconvert.

Materials and methods

Azotobacter chroococcum (N.C.I.B. 8003) was cultured and harvested as described previously (Yates, 1970) and stored in liquid N_2. *A. chroococcum* Fd was isolated as described for *A. vinelandii* Fd I under fully aerobic conditions (Yoch *et al.*, 1969). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis at pH7.4 indicated that the protein was 90% pure. The absorption spectrum was similar to that reported for *A. vinelandii* FdI (Yoch *et al.*, 1969). Ferrous sulphate (BDH) was recrystallized three times from water. Ethanediol and $Na_2S_2O_4$ were obtained from BDH. Pipes and Taps were from Sigma.

Molar concentrations of solution were obtained by absorption spectroscopy using $\varepsilon_{400} = 29800\,M^{-1}\cdot cm^{-1}$ (Morgan *et al.*, 1984*b*). Reduction of the protein was carried out anaerobically inside an O_2-free N_2-flushed glove box (Faircrest Engineering; $O_2 < 1$ p.p.m.) using carefully deoxygenated solutions and solutions of 0.1M-$Na_2S_2O_4$. For low-temperature m.c.d. experiments the glassing agent deoxygenated ethanediol was added to the protein solution to give a final concentration of 50% (v/v). It is important to monitor the pH closely during the addition of ethanediol and, if necessary, to adjust for pH changes. The buffers Taps and Pipes used throughout this work are zwitterions which are known to exhibit minimal pH shifts on cooling (Williams-Smith *et al.*, 1977). The pH titration of the $Na_2S_2O_4$-reduced Fd was carried out in the following way. Two stock solutions of protein, equimolar and in 20mM-Pipes and 20mM-Taps were prepared. An aliquot of each solution was taken and its pH adjusted by addition of 1M-Pipes or -Taps buffer solution, respectively. The ionic strength was maintained constant at 0.18M throughout. In this way it was possible to cover the pH range 6.1–7.5 with Pipes and 7.7–9.1 with Taps. Ethanediol was added and the pH measured. This value was used for the pK_a determination.

Absorption spectra were measured using a Cary 17 or a Unicam SP-8.400 spectrophotometer. C.d. and m.c.d. spectra were obtained with a Jasco J500D spectropolarimeter, interfaced to a Commodore 710B microcomputer (S. J. George, unpublished work), and with a split-coil, top-loading superconducting solenoid (SM-4; Oxford Instruments), capable of generating magnetic fields up to 5T and giving sample temperature control between 1.5 and 300K. Optical cells which maintain a solution anaerobic were filled inside the glove box and inserted into the liquid He of the magnet system. E.p.r. spectra were obtained on solutions taken from the same stock as the m.c.d. samples and frozen to liquid-N_2 temperature in quartz tubes within the glove box. The e.p.r. instrument was the Bruker ER-200 C, interfaced to an Aspect 2000 computer and fitted with an ESR-9 flow cryostat (Oxford Instruments). Integration of e.p.r. signals was carried out using the $g_z = 3.45$ peak of metmyoglobin–cyanide as a standard (Aasa & Vänngård, 1975). M.c.d. spectra are quoted in units of $\Delta\varepsilon$ ($= \varepsilon_L - \varepsilon_R$), $M^{-1}\cdot cm^{-1}$, not normalized for magnetic field.

Results and discussion

The room temperature c.d. spectrum and the m.c.d. spectrum at 4.2K (Johnson *et al.*, 1984) of the Fd as aerobically isolated from *A. chroococcum* are identical with those of *A. vinelandii* Fd I (Morgan *et al.*, 1984*a*,*b*). Similarly the e.p.r. spectra of the two proteins are indistingishable. An e.p.r. study of the *A. chroococcum* protein (R. A. Cammack & M. G. Yates, unpublished work) shows the development with hexacyanoferrate oxidation of signals identical with those reported by Morgan *et al.* (1984*b*) for *A. vinelandii*. These results establish clearly that the clusters in the proteins from the two sources are identical spectroscopically.

Reduction of *A. chroococcum* Fd with $Na_2S_2O_4$ leads to two distinctly different m.c.d. spectra, RI and RII, depending upon the pH of the buffer solution. Fig. 1 shows the m.c.d. spectra at 4.2K and 5T of $Na_2S_2O_4$-reduced Fd at pH values of 6.3, 7.4 and 8.3. The samples are in the presence of excess EDTA in order to scavenge any adventi-

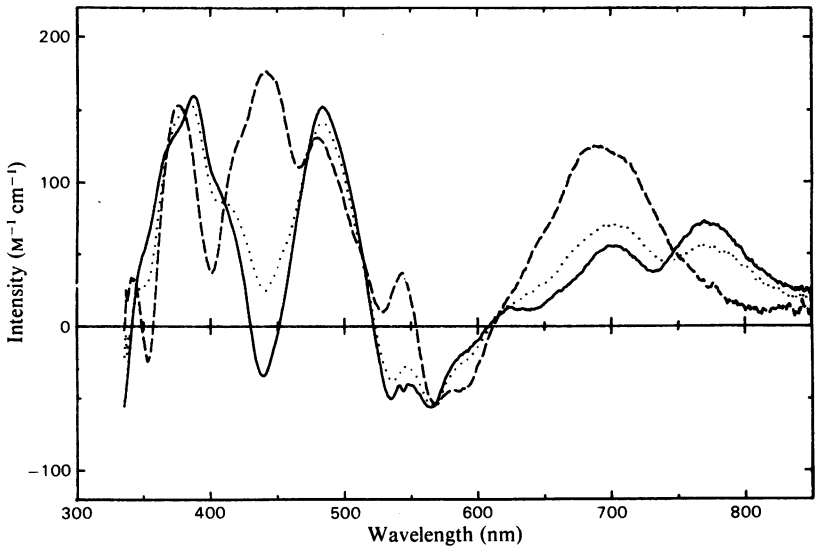

Fig. 1. *Low temperature m.c.d. spectra of $Na_2S_2O_4$-reduced A. chroococcum Fd*
———, 165 μM-Fd in 90 mM-Pipes, pH 6.3; ·····, 165 μM-Fd in 90 mM-Pipes, pH 7.4; – – – –, 150 μM-Fd in 90 mM-Taps, pH 8.3. All were diluted with ethanediol (1:1, v/v) and contain EDTA. Concentrations quoted are after dilution. All spectra were run at 4.2 K and 5.0 T.

tious iron in solution (see below). The e.p.r. spectra of all of these samples are devoid of any significant signals. However, the m.c.d. spectra are temperature-dependent, showing that they arise from a paramagnetic species. Preliminary analyses of the m.c.d. magnetization curves of RI and RII indicate that both have spin states $S = 2$ with negative axial zero-field splitting parameters (Thomson *et al.*, 1981). Therefore the spectra in Fig. 1 arise entirely from the reduced 3Fe cluster.

The m.c.d. spectrum characteristic of RI has been observed in Pipes buffer over the pH range 6.3–7.4 and that of RII in Taps over the pH range 7.9–9.2. Interconversion of the two species takes place between pH 7.0 and 8.0, indicating a pK_a of ~7.5. RI and RII have been interconverted in both directions. Fd has been cycled between pH values of 6.3 and 9.2 both in the oxidized and in the $Na_2S_2O_4$-reduced states. The m.c.d. spectrum of the oxidized state is invariant with pH over this range. The form of the $Na_2S_2O_4$-reduced spectrum does not depend upon the previous pH or redox history of the sample. Therefore we conclude that the two reduced forms of the 3Fe cluster, RI and RII, are reversibly interconvertible with a $pK_a \sim 7.5$.

The form of the m.c.d. spectrum at pH 8.3, RII, is very similar to that of the $Na_2S_2O_4$-reduced [3Fe-4S] cluster in *D. gigas* FdII (Thomson *et al.*, 1981) and in beef heart aconitase (Johnson *et al.*, 1984) (Fig. 2). The m.c.d. magnetization properties, which are a sensitive indicator of the electron

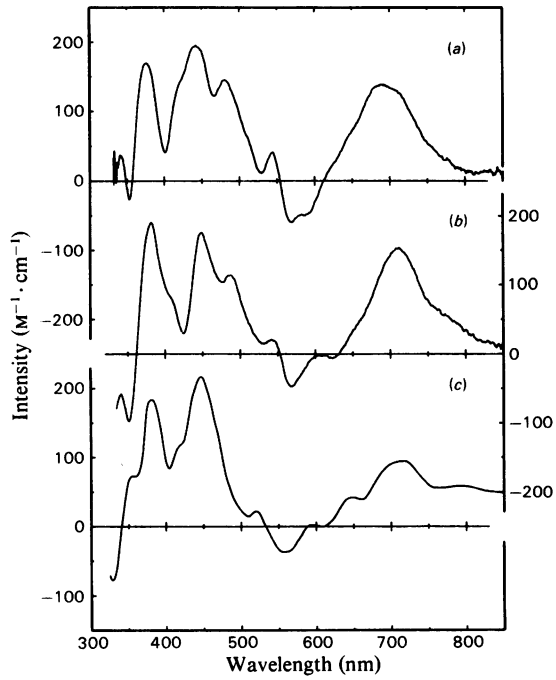

Fig. 2. *Low-temperature m.c.d. spectra of dithionite-reduced [3Fe-xS]-containing proteins*
(*a*), 150 μM *A. chroococcum* Fd in 90 mM-Taps, pH 8.3; (*b*), 200 μM *D. gigas* Fd II in 25 mM-Pipes, pH 6.5; (*c*), 81 μM ox heart aconitase in 25 mM-Tris, pH 8.5. All samples were diluted with ethanediol (1:1, v/v). All concentrations are quoted after dilution. Spectra were recorded at 1.6 K and 5.0 T.

spin and zero-field splitting parameters of the ground state, are identical in form at all wavelengths for these three proteins. These facts show that the reduced 3Fe clusters in *A. chroococcum* Fd RII, *D. gigas* FdII and aconitase are very similar and therefore all have the core stoichiometry of [3Fe-4S]. The m.c.d. spectrum of the $Na_2S_2O_4$-reduced form of the *A. chroococcum* Fd at pH 6.5, RI, is distinctly different from that of the high-pH form and from that of any other proteins so far reported. This species is not generated in $Na_2S_2O_4$-reduced *D. gigas* FdII at pH 6.4 (S. J. George, A. J. M. Richards, A. J. Thomson, A. V. Xavier, J. J. G. Moura, I. Moura & J. LeGall, unpublished work).

The nature of the structural change undergone by the reduced 3Fe cluster is not established by our studies. However, the reversibility of the RI↔RII interconversion makes it unlikely that a sulphide ion, either S^{2-} or SH^-, is being removed from the cluster core. Two possibilities are, first, that the tri-capping apical sulphide ion of the [3Fe-4S] core can become protonated or, secondly, that the number of cysteine ligands binding the [3Fe-4S] core depends upon pH.

$Na_2S_2O_4$ reduction of *A. chroococcum* Fd in 50mM-Pipes, pH 8.9, after incubation for 15min with a 2-fold excess of $FeSO_4$ over protein leads to an e.p.r. spectrum (Fig. 3) with *g*-values of 2.04 and 1.93. Integration of this signal using metmyoglobin–cyanide as a standard gives a value of 1 electron spin/mol of protein. The m.c.d. spectrum of the sample sample at 1.6K and 5T is also shown in Fig. 3. The m.c.d. magnetization properties (not shown) of the bands in the spectrum show that they all arise from a paramagnet with a spin $S = \frac{1}{2}$ and a *g*-value close to 2.0. Hence there is no contribution to the m.c.d. spectrum from a reduced 3Fe cluster, which gives m.c.d. magnetization curves much steeper than those of $S = \frac{1}{2}$, $g = 2$ paramagnets (Thomson *et al.*, 1981). The e.p.r. spectrum has the form expected for a simple $[4Fe\text{-}4S]^{1+}$ cluster. It is axial, as are the e.p.r. signals of inorganic models such as $(Et_4N)_3[Fe_4S_4(SCH_2\text{-}C_6H_5)_4]$ (Laskowski *et al.*, 1979). The additional features seen in the e.p.r. spectrum of a fully reduced 8-Fe ferredoxin such as that from *Micrococcus lactilyticus* (Mathews *et al.*, 1974), which are due to spin-coupling between clusters, are absent from Fig. 3. For this reason and because of the spin integration there must be at most one reduced [4Fe-4S] cluster. The form of the m.c.d. spectrum is similar to, but not identical with, that for the reduced $[4Fe\text{-}4S]^{1+}$ cluster in *Clostridum pasteurianum* Fd (Johnson *et al.*, 1981) and in *Desulfovibrio africanus* Fd I and II (Hatchikian *et al.*, 1984). Therefore we assign the spectra of Fig. 3 to a semi-reduced 8Fe ferredoxin ($[4Fe\text{-}4S]^{1+}[4Fe\text{-}4S]^{2+}$) and conclude that the 3Fe cluster has been transformed into a [4Fe-4S] cluster by $Na_2S_2O_4$ reduction in the presence of ferrous ion. In some preparations we have observed the formation of the same e.p.r. signal and m.c.d. spectrum on $Na_2S_2O_4$ reduction at pH values as

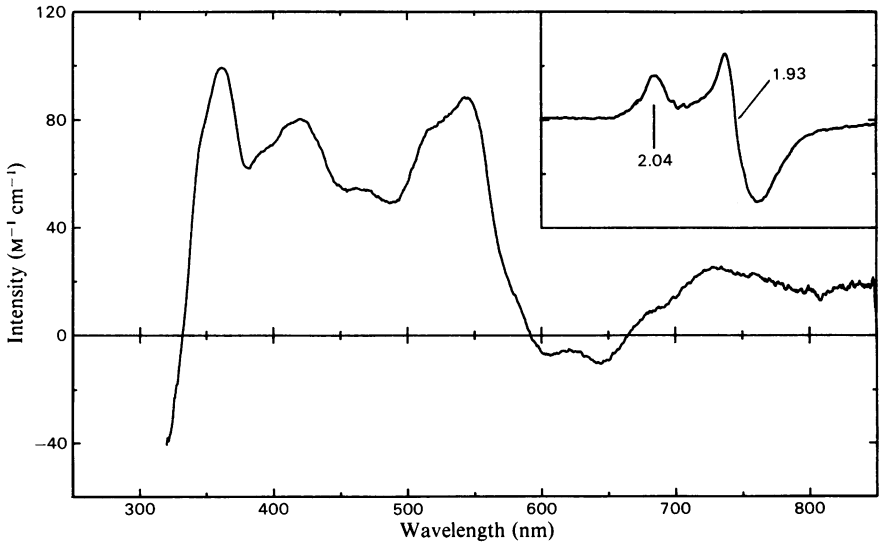

Fig. 3. *Low-temperature m.c.d. spectra of dithionite-reduced A. chroococcum Fd*
Sample was 150 μM in 25mM-Taps, pH 8.9, with a 2-fold excess of $FeSO_4$ present and in water/ethanediol (1:1, v/v). The spectrum was run at 1.6K and 5T. Insert, X-band e.p.r. spectrum of the same sample. Temperature 10K, microwave frequency 9.21GHz, power 2mW, modulation amplitude 5G.

low as 7.5 without the addition of ferrous ion. Beinert and co-workers (Kennedy *et al.*, 1983) have shown that $Na_2S_2O_4$ reduction of ox heart aconitase leads to the conversion of the [3Fe-4S] cluster to a [4Fe-4S] cluster. It was further shown that the presence of iron chelators such as EDTA in the reduction mixture inhibited the build-up of the 4Fe cluster. We have also observed that the 3Fe cluster in *A. chroococcum* Fd is prevented from forming a 4Fe cluster by the presence of EDTA. Indeed, in some preparations it is difficult to obtain m.c.d. spectra of RII totally free from a contribution from $[4Fe\text{-}4S]^{1+}$ cluster spectra without the presence of EDTA. For this reason we carried out the pH titration shown in Fig. 1 in the presence of EDTA.

The extent of conversion of [3Fe-4S] to [4Fe-4S] depends upon the length of reduction with $Na_2S_2O_4$, upon the amount of adventitious iron available and upon the pH. We have never observed the formation of the $[4Fe\text{-}4S]^{1+}$ cluster at pH 6.5. This suggests the possibility that there is in *A. chroococcum* Fd a pH switch operating at pH 7.5 that controls in the reduced state of the protein, whether it is a 7Fe or 8Fe ferredoxin. A recent report of the total anaerobic reconstitution of the apoprotein of *A. vinelandii* Fd I shows that a semi-reduced 8Fe and oxidized 8Fe Fd is formed directly (Morgan *et al.*, 1984*a*). These findings also raise interesting questions about the biological activity of the two reduced states of the 3Fe cluster and of the semi-reduced form of the 8Fe ferredoxin.

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